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First record of *Scopula orientalis* (Alphéraky, 1876) (Lepidoptera, Geometridae) in Romania, at the northern limit of the Balkans

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Abstract. The geometrid moth *Scopula orientalis* (Alphéraky, 1876) has an apparently disjunct distribution in Europe, with local populations in the Balkans (Macedonia, Bulgaria and Albania where recently discovered), as well as in Ukraine and southern European Russia. In this study, based on morphological and mitochondrial DNA (cytochrome c oxidase subunit 1 – COI) data, we report the presence of *S. orientalis* in south-eastern Romania (Dobrogea), at the northernmost limit of the Balkans. The flight time (September) of the recorded specimen, is the latest seasonal record for the Balkans, supporting the presence of at least a partial second generation. The Romanian specimen represented a unique COI haplotype which is differentiated by seven mutations from its genetically closest population in Sivas province, Turkey. The presence of *S. orientalis* in Romania reduces the distributional gap between the known Balkan and Ukrainian populations and highlights the potential for a more widespread distribution that needs documentation based on directed studies.

Introduction

The *ornata* species-group of the genus *Scopula* Schrank, 1802 (family Geometridae) is represented in Europe by nine species (Hausmann 2004; Can 2009). Among these, *Scopula orientalis* (Alphéraky, 1876) has a disjunct distribution with isolated populations in the Balkans, then in Ukraine, southern European Russia and southern Urals (Hausmann 2004; Beshkov 2017). Outside Europe, the species occurs in Turkey, Caucasus, Transcaucasus and northern Iran, apparently reaching the mountains of central Asia (Viidalepp 1996; Hausmann 2004). A large part of the European distribution gap of this species is situated in the Romanian territory, between the northern limit of the Balkans and Ukraine. In Romania, the *ornata* species-group is currently known to be represented by only two species: *Scopula ornata* (Scopoli, 1763) and *Scopula decorata* ([Denis & Schiffermüller], 1775) (Rákosy et al. 2003; Rákosy and Goia 2006), both externally similar to *S. orientalis*.

In this study, based on genitalia morphology and DNA data, we report the presence of *S. orientalis* in the Romanian fauna.

Methods

Morphological analyses

We examined the genitalic morphology of 24 specimens (1 Bulgarian, 1 Spanish, 2 Italian and 20 Romanian) of the *ornata* species-group (Suppl. material 1).

The male and female genitalia were processed as follows: maceration in 10% potassium hydroxide (ca. six minutes at 90 °C), cleaning in distilled water, examination under a stereomicroscope and storage in tubes with glycerine. Photos of the genitalia were taken in 70% ethanol by using a digital camera attached to a stereomicroscope. Specimens were identified based on comparison with illustrations and text from Hausmann (2004).

DNA sequencing

From the 16 specimens sequenced during this study, total genomic DNA was extracted using Chelex 100 resin, 100–200 mesh, sodium form (Biorad), under the following protocol: one leg was removed and introduced into 100 µl of Chelex 10% and 5 µl of Proteinase K (20 mg/ml) were added. The samples were incubated overnight at 55 °C and were subsequently incubated at 100 °C for 15 min. Samples were then centrifuged for 10 s at 3000 rpm. A 658-bp fragment near the 5′ end of COI was amplified by polymerase chain reaction using the primers LepF1 (5′-ATTCAACCAATCATAAAGATATTGG-3′) and LepR1 (5′- TAAACTTCTGGATGTCCAAAAAATCA-3′) (Hebert et al. 2004). Double-stranded DNA was amplified in 25-µl volume reactions containing: 14.4 µl autoclaved Milli-Q water, 5 µl 5x buffer, 2 µl 25 mM MgCl2, 0.5 µl 10 mM dNTPs, 0.5 µl of each primer (10 µM), 0.1 µl Taq DNA Polymerase (Promega, 5U/µl) and 2 µl of extracted DNA. The typical thermal cycling profile followed this protocol: first denaturation at 92 °C for 60 s, followed by five cycles of 92 °C for 15 s, 48 °C for 45 s and 62 °C for 150 s, and then by 35 cycles of 92 °C for 15 s, 52 °C for 45 s and 62 °C for 150 s and a final extension at 62 °C for 420 s. PCR products were purified and sequenced by Macrogen Inc.

Sequences were edited and aligned using GENEIOUS PRO 6.1.8 created by Biomatters (http://www.geneious.com/). The 16 sequences obtained in this study have been submitted to GenBank (Suppl. material 1) and are also publicly available in the dataset DS-SCOP (dx.doi.org/10.5883/DS-SCOP) from the Barcode of Life Data Systems (http://www.boldsystems.org/).

Analyses of DNA sequences

The DNA analyses included 90 cytochrome *c* oxidase subunit 1 (COI) sequences. Seventy-one sequences represented all species of the European *ornata* species-group available in GenBank (six out of nine species present in Europe) and originated from Õunap et al. (2008) (two sequences), Can (2009) (11 sequences), Huemer and Hebert (2012) (one sequence) and Hausmann et al. (2013) (57 sequences). Sixteen additional sequences were obtained for this study (12 *S. decorata*, three *S. ornata* and one *S. orientalis*). *Scopula rubiginata* Hufnagel, 1767, *Thetidia smaragdaria* Fabricius, 1787 and *Rhodostrophia vibicaria* Clerck, 1759) were added to the dataset as outgroups (Suppl. material 1).

For DNA-based identifications, a neighbour-joining (NJ) tree was constructed in MEGA7 (Kumar et al. 2016), using an alignment comprising 90 COI sequences. The genetic distances were computed implementing the p-distance method (Nei and Kumar 2000), using pairwise deletion and node supports were assessed with 100 bootstrap replicates.

Results

Morphology-based results

The genitalic examination of 21 specimens (1 Bulgarian, 20 Romanian) of the *ornata* species-group indicated that 18 represented *S. decorata*, two *S. ornata* and one *S. orientalis* (Suppl. material 1). The single specimen of *S. orientalis* originated from south-eastern Romania (northern Dobrogea) (Fig. 1).

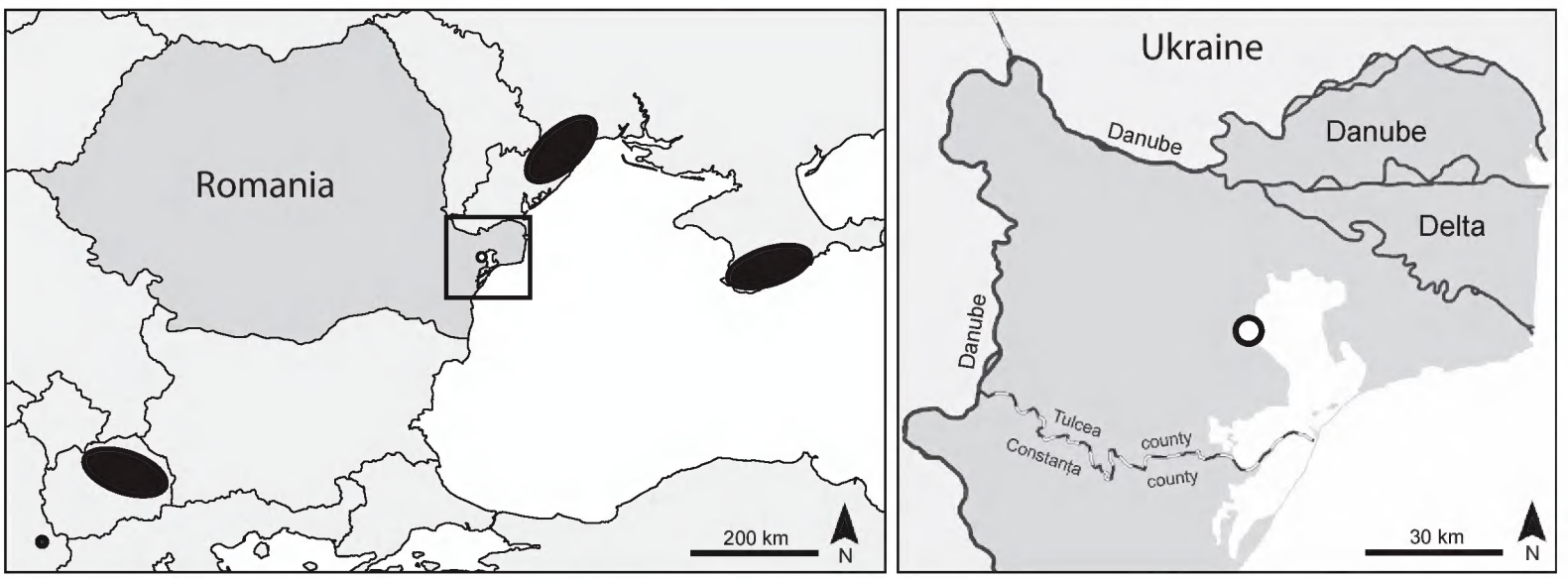

Figure 1. Left panel – geographic location of the Romanian record of *S. orientalis* and the nearest known areas of occurence for this species (black ovals and dot). Right panel – detail of the location (black circle) of the Romanian record of *S. orientalis*, Enisala fortress, Tulcea county.

Material. *S. orientalis*, 1 ♂, Enisala, Tulcea county (northern Dobrogea), Romania, 4.ix.2015, 85m (44° 53.01′N, 28° 50.10′E), leg. & coll. L. Székely. Prep. genit. 2318/Dincă, COI GenBank accesion number MG786139. Wing span 22 mm (Fig. 2, Suppl. material 1).

Scopula orientalis (Fig. 2) is externally similar especially to *S. ornata* and *S. decorata*, from which it differs through rather subtle morphological characters (see Hausmann 2004). The male and female genitalia of *S. orientalis* are fairly similar to those of the Iberian endemic *Scopula concinnaria* (Duponchel, 1842), but allow a reliable separation from *S. ornata* and *S. decorata* (Hausmann 2004). The male specimen originating from Romania displays genitalia characteristics typical of *S. orientalis* (Fig. 3a–c), and can be readily distinguished from *S. decorata* (Fig. 3d) and *S. ornata* (Fig. 3e) especially through the shape of the sternum A8 and its stout cerata (the left one more curved), both with strongly spinose tips.

DNA results

The NJ analysis showed that each species of the *ornata* species group formed a well-supported clade (Fig. 4), confirming the ability of DNA barcodes to distinguish taxa of this species-group (Can 2009; Hausmann et al. 2013).

The clade formed by *S. orientalis* included the Romanian specimen that was also identified based on genitalic morphology. This specimen represented a unique haplotype diverging by seven mutations (ca 1%) from conspecific specimens from Sivas province (Turkey) and by 15 mutations (ca 2.3%) from the specimen from Gümüşhane (Fig. 4).

Figure 2. Male of *S. orientalis* from Romania, Enisala fortress, Tulcea county, 4.ix.2015, prep. genit. 2318/Dincă. Scale bar is 10 mm. Photo: V. Dincă.

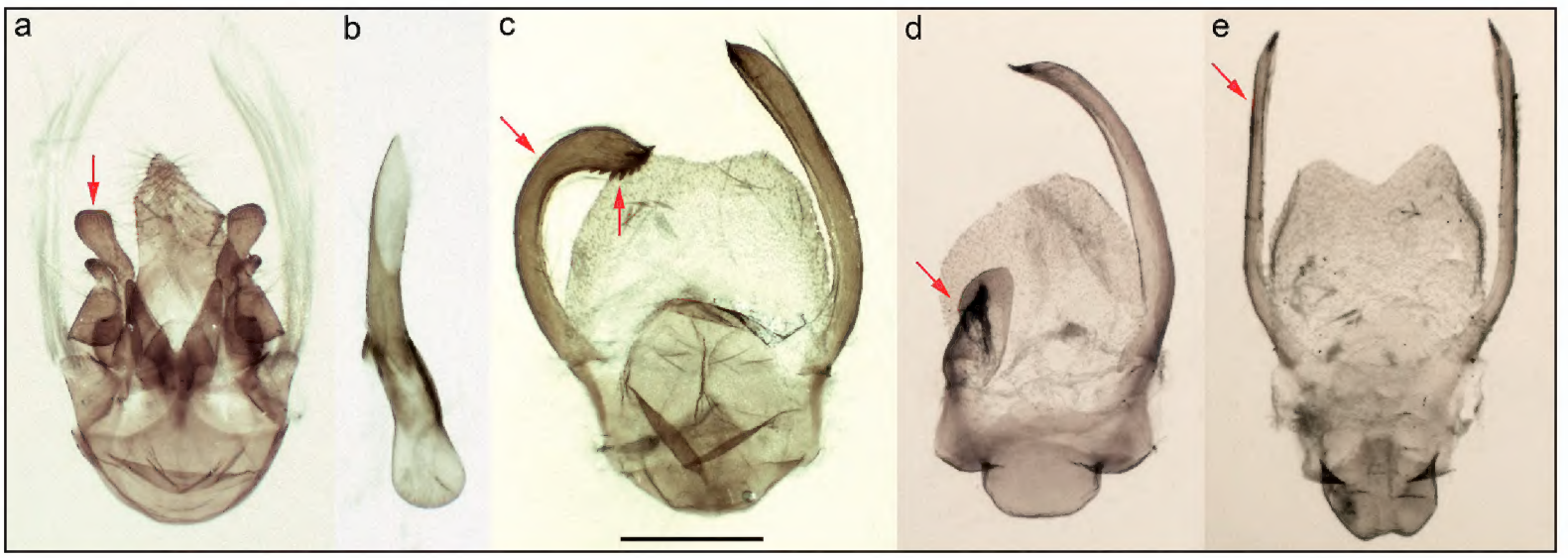

Figure 3. Male genitalia elements of *S. orientalis*, *S. decorata* and *S. ornata*. **a-c.** Male genitalia of *S. orientalis* from Romania (Enisala fortress, Tulcea county, 4.ix.2015), prep. genit. 2318/Dincă. **a.** Genital armature. **b.** Aedeagus. **c.** Sternum A8. **d.** Sternum A8 of male *S. decorata*, Romania (Enisala fortress, Tulcea county, 10.vii.2013), prep. genit. 2405/Dincă. **e.** Sternum A8 of male *S. ornata*, Romania (Enisala fortress, Tulcea county, 24.viii.2016), prep. genit. 2417/Dincă. Scale bar is 0.5 mm.

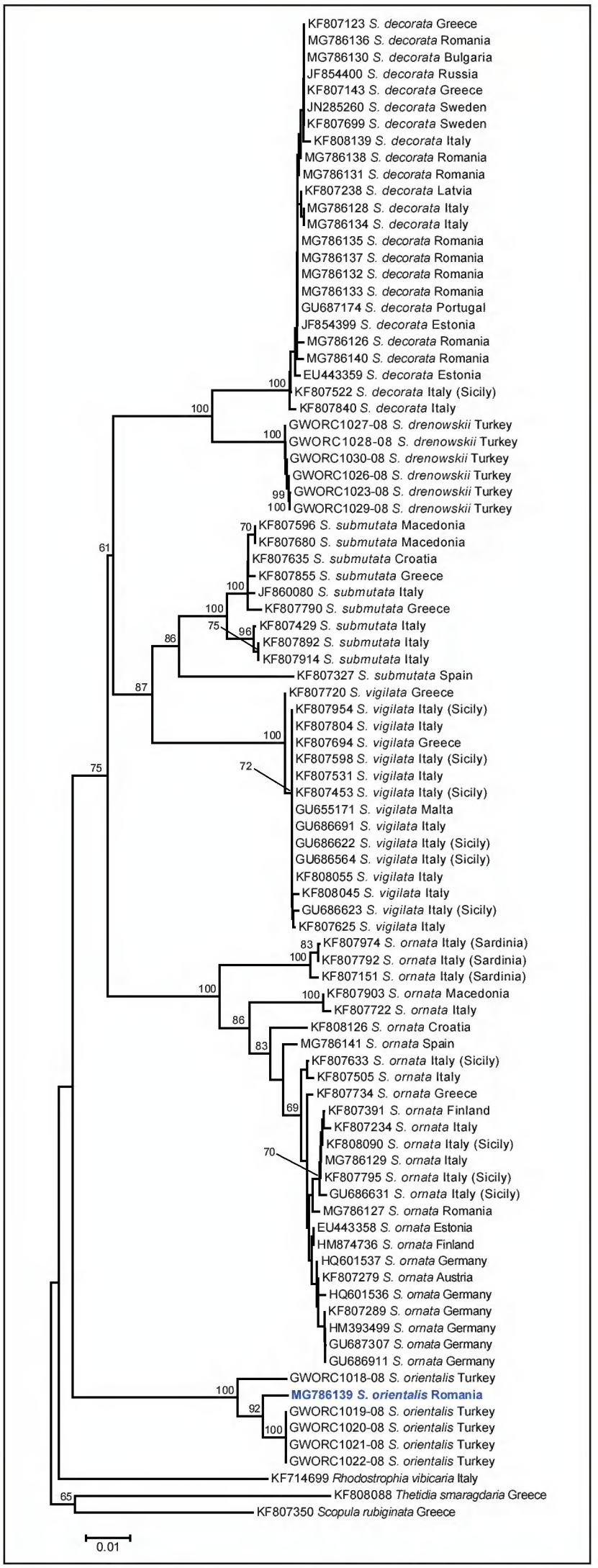
KF807123 S. decorata Greece
MG786136 S. decorata Romania
MG786130 S. decorata Bulgaria
JF854400 S. decorata Russia
KF807143 S. decorata Greece
JN285260 S. decorata Sweden
KF807699 S. decorata Sweden
KF808139 S. decorata Italy
MG786138 S. decorata Romania
MG786131 S. decorata Romania
KF807238 S. decorata Latvia
MG786128 S. decorata Italy
MG786134 S. decorata Italy
MG786135 S. decorata Romania
MG786137 S. decorata Romania
MG786132 S. decorata Romania
MG786133 S. decorata Romania
GU687174 S. decorata Portugal
JF854399 S. decorata Estonia
MG786126 S. decorata Romania
MG786140 S. decorata Romania
EU443359 S. decorata Estonia
KF807522 S. decorata Italy (Sicily)
KF807840 S. decorata Italy
GWORC1027-08 S. drenowskii Turkey
GWORC1028-08 S. drenowskii Turkey
GWORC1030-08 S. drenowskii Turkey
GWORC1026-08 S. drenowskii Turkey
GWORC1023-08 S. drenowskii Turkey
GWORC1029-08 S. drenowskii Turkey
KF807596 S. submutata Macedonia
KF807680 S. submutata Macedonia
KF807635 S. submutata Croatia
KF807855 S. submutata Greece
JF860080 S. submutata Italy
KF807790 S. submutata Greece
KF807429 S. submutata Italy
KF807892 S. submutata Italy
KF807914 S. submutata Italy
KF807327 S. submutata Spain
KF807720 S. vigilata Greece
KF807954 S. vigilata Italy (Sicily)
KF807804 S. vigilata Italy
KF807694 S. vigilata Greece
KF807598 S. vigilata Italy (Sicily)
KF807531 S. vigilata Italy
KF807453 S. vigilata Italy (Sicily)
GU655171 S. vigilata Malta
GU686691 S. vigilata Italy
GU686622 S. vigilata Italy (Sicily)
GU686564 S. vigilata Italy (Sicily)
KF808055 S. vigilata Italy
KF808045 S. vigilata Italy
GU686623 S. vigilata Italy (Sicily)
KF807625 S. vigilata Italy
KF807974 S. ornata Italy (Sardinia)
KF807792 S. ornata Italy (Sardinia)
KF807151 S. ornata Italy (Sardinia)
KF807903 S. ornata Macedonia
KF807722 S. ornata Italy
KF808126 S. ornata Croatia
MG786141 S. ornata Spain
KF807633 S. ornata Italy (Sicily)
KF807505 S. ornata Italy
KF807734 S. ornata Greece
KF807391 S. ornata Finland
KF807234 S. ornata Italy
KF808090 S. ornata Italy (Sicily)
MG786129 S. ornata Italy
KF807795 S. ornata Italy (Sicily)
GU686631 S. ornata Italy (Sicily)
MG786127 S. ornata Romania
EU443358 S. ornata Estonia
HM874736 S. ornata Finland
HQ601537 S. ornata Germany
KF807279 S. ornata Austria
HQ601536 S. ornata Germany
KF807289 S. ornata Germany
HM393499 S. ornata Germany
GU687307 S. ornata Germany
GU686911 S. ornata Germany
GWORC1018-08 S. orientalis Turkey
MG786139 S. orientalis Romania
GWORC1019-08 S. orientalis Turkey
GWORC1020-08 S. orientalis Turkey
GWORC1021-08 S. orientalis Turkey
GWORC1022-08 S. orientalis Turkey
KF714699 Rhodostrophia vibicaria Italy
KF808088 Thetidia smaragdaria Greece
KF807350 Scopula rubiginata Greece
0.01

Figure 4. Neighbor-joining tree based on COI sequences highlighting (bold blue) the position of the Romanian specimen of *S. orientalis*. Bootstrap supports (>60) are shown next to recovered nodes.

Discussion

Scopula orientalis was described as a variety of *S. decorata* (*Acidalia decorata* var. *orientalis*), based on material originating from south-western European Russia (Alphéraky 1876). The taxon was raised to species rank only in 1931 when Obraztsov illustrated the genital armature of the species for the first time (Obraztsov 1931).

The presence of *S. orientalis* in the Romanian fauna is not unexpected, as it narrows the gap between the known populations in the Balkans and those in Ukraine. Since only one record is available, we cannot completely rule out the possibility that our specimen represents a stray individual originating from other areas. However, this hypothesis is unlikely because *S. orientalis* is a weak flyer and we have not found any notes describing migratory behaviour. Furthermore, the habitat from Enisala fortress (Fig. 5) appears suitable for the species and the available specimen is fresh (Fig. 2). The nearest populations to the Romanian sampling site are those from Odess'ka oblast in southern Ukraine, approximately 160 km north-east from Enisala fortress (I. Kostjuk pers. comm. to L. Székely) (Fig. 1). In Ukraine the species is localized, and has sporadically been reported from the oblasts Odess'ka, Mykolaivs'ka, Kirovohrads'ka, Zaporizzs'ka, Donets'ka and Luhans'ka, as well as Crimea (Budashkin and Kostjuk 1987; Budashkin 2004; Kostjuk 2004; I. Kostjuk pers. comm. to L. Székely). *Scopula orientalis* is locally common in Crimea (V. Savchuk, pers. comm. to L. Székely).

In the Balkans, the few confirmed records of this species originate from Macedonia, south-western Bulgaria and south-eastern Albania (Hausmann 2004; Beshkov 2017; Beshkov pers. comm. 2017 for Bulgaria), at least 550 km away from the Romanian locality reported here (Fig. 1).

The data above show that *S. orientalis* is a species with very poorly known distribution in Europe, especially in the Balkans. The lack of data is probably due both to the local character of the species, but also to the fact that it has been overlooked due to its external similarity to other *Scopula* species, particularly *S. ornata* and *S. decorata*.

The larval food plant of *S. orientalis* is unknown and detailed information on habitat preferences is very limited (Hausmann 2004; Beshkov 2017). The single known Albanian specimen was captured along a river valley with steep stony slopes partially covered by thermophilous species of trees and shrubs (Beshkov 2017). The Romanian specimen was attracted to an automatic light trap placed in a rocky (limestone) open steppe area (Fig. 5a, b), at approximately 85m a.s.l. This hilly area is bordered by vast wetlands (Fig. 5a, b), generating contrasting habitats with specific flora and fauna.

Scopula orientalis is considered to be usually univoltine (mid-May to end July, depending on altitude) (e.g., Obraztsov 1936; Hausmann 2004), but one record from early September (Armenia) suggests that at least a partial second generation may exist in some areas (Hausmann 2004). This is also suggested by records from Ukraine, which range from May to August (I. Kostjuk pers. comm. to L. Székely). The Romanian record further supports this possibility, since the male specimen, likely recently emerged (Fig. 2), was collected on 4 September. Interestingly, none of the *ornata* species-group specimens from the same site (Enisala fortress) sampled between the beginning of June and the end of August belonged to *S. orientalis* (Suppl. material 1). More detailed research is undoubtedly needed to clarify the phenology of this species in northern Dobrogea and in Europe in general.

The Romanian specimen of *S. orientalis* is also among the smallest reported for this species: its 22 mm wing span has been reported only for Turkish specimens, while the usual size is apparently

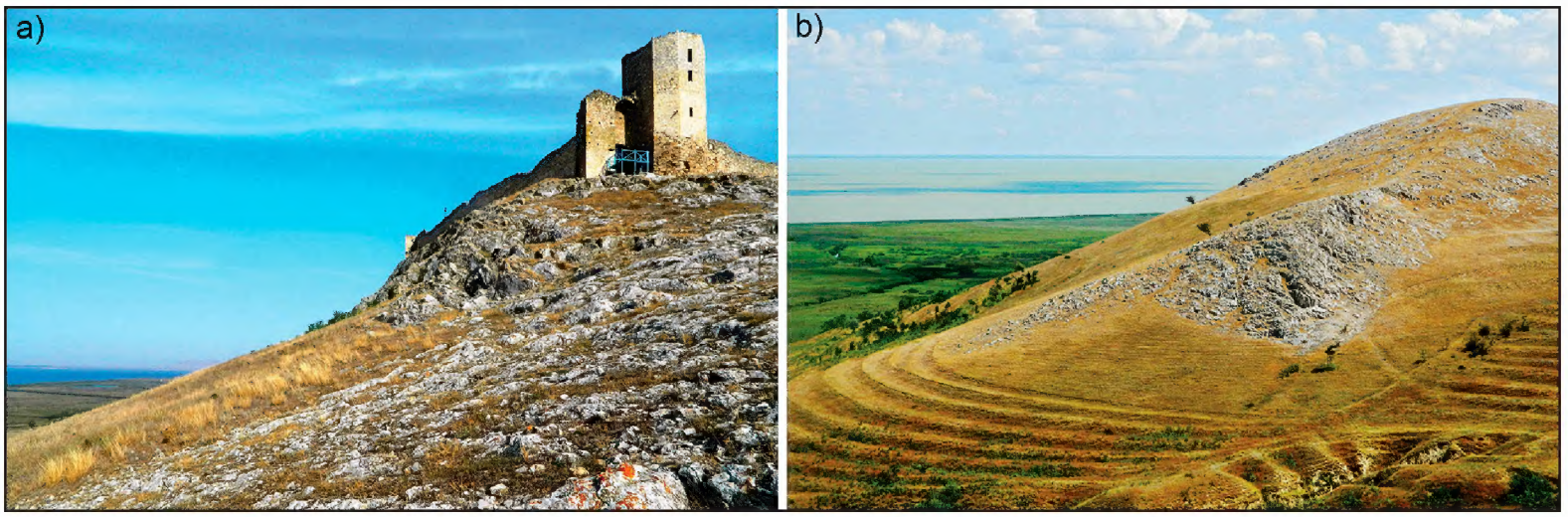

Figure 5. Collecting site of *Scopula orientalis* in Romania. **a.** Rocky steppe slopes at Enisala fortress, Tulcea county (photo taken on 30.ix.2017). **b.** The dry slopes around Enisala fortress, were *S. orientalis* was collected, are bordered by vast wetland areas, which are partly visible on the left side of the image (photo taken on 26.viii.2016). Photos: L. Székely.

between 24–29 mm (Hausmann 2004). The recently found Albanian specimen had a wingspan of 30 mm (Beshkov 2017). Without additional material it is not possible to determine whether this small size is a characteristic of the Romanian population, but it can be hypothesized that the extreme rocky steppe habitat at low elevation and/or late flight time played a significant role in this respect.

The mitochondrial DNA (mtDNA) data available have shown that the species of the *ornata* species-group can be reliably identified based on DNA barcodes, and confirmed the morphology-based identification of *S. orientalis* from Romania (Fig. 4). Within *S. orientalis*, it is also worth noting that Turkish specimens from the provinces of Sivas and Gümüşhane, separated by ca 250 km, are genetically much more differentiated than are specimens from Sivas and Romania, separated by almost 900 km (Fig. 4, Suppl. material 1). The current sampling is certainly too limited to draw any conclusions, but it suggests that the Romanian population may have been isolated for a considerable amount of time. Such patterns highlight the need for further study using refined sampling and additional sources of data to clarify the phylogeography and evolutionary history of the taxa belonging to the *ornata* species-group.

Although *S. orientalis* is not protected in Europe, the site where this species was found in Romania (Enisala fortress) lies within the territory of the Danube Delta Biosphere Reserve. The rocky steppe habitat lies at the base of Enisala fortress and should be relatively safe from human intervention. This area harbours a series of Lepidoptera species of high zoogeographical value and with extremely limited distributions in Romania and/or Europe (Székely 2016). In the steppe areas, such examples are *Lemonia balcanica* (Herrich-Schäffer, 1847), *Eupithecia biornata* Christoph, 1867, *Cervyna cervago* (Eversmann, 1844), *Cucullia santonici* (Hübner, [1813]), *Eublemma porphyrina* (Freyer, 1844), while the neighbouring wetlands are optimal habitats for taxa such as *Diachrysia chryson deltaica* Rákosy, 1996 and *Rhyparioides metelkana* (Lederer, 1861).

In conclusion, *Scopula orientalis* is currently known in Romania only from Enisala fortress, where only one specimen has been found. This situation may nevertheless change if directed research is done (i.e. through dedicated field work, but also re-examination of *ornata* species-group material stored in collections). Such research could also reveal the presence of the species in other regions of southern Romania, including of course other parts of Dobrogea. For example, if the spe-

cies is found in southern Dobrogea, this would suggest the presence of *S. orientalis* in north-eastern Bulgaria as well. The record from Enisala fortress diminishes the geographic gap between the populations from the Balkans and those from southern Ukraine, and also suggests that *S. orientalis* may be present in the Republic of Moldavia (Fig. 2).

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Supplementary material 1

Table S1. Specimens and DNA sequences used in this study

Authors: Vlad Dincă, Levente Székely

Data type: Adobe PDF file

Explanation note: No cytochrome c oxidase subunit 1 (COI) sequences were available in GenBank for the following three species of the ornata species-group: *Scopula concinnaria*, *S. honestata* and *S. subtilata*.

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